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Antibacterial activity of ozonized sunflower oil (Oleozon)

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Aims: To evaluate the antimicrobial effect of the ozonized sunflower oil (Oleozon) on different bacterial species isolated from different sites.

Methods and Results: The effect of Oleozon on Mycobacteria, staphylococci, streptococci, enterococci, *Pseudomonas* and *Escherichia coli* was tested. The sunflower oil was ozonized at the Centro de Investigaciones del Ozone (CENIC, Havana, Cuba) by an ozone generator. MICs were determined by the agar dilution method. For Mycobacteria, the MIC of Oleozon was determined on solid medium by a microdrop agar proportion test. Oleozon showed antimicrobial activity against all strains analysed, with an MIC ranging from 1·18 to 9·5 mg ml^{-1}.

Conclusions: Oleozon showed a valuable antimicrobial activity against all micro-organisms tested. Results suggest that Mycobacteria are more susceptible to Oleozon than the other bacteria tested.

Significance and Impact of the Study: The wide availability of sunflower oil makes Oleozon a competitive antimicrobial agent. These results should prompt the setting up of some clinical trials to compare Oleozon with other antimicrobial agents.

INTRODUCTION

Ozone is a powerful oxidant, principally applied as a disinfectant of drinking and waste water (Alvarez and O'Brien 1982; Vanden Bossche *et al*. 1994; Gundarova *et al*. 1996; Legnani *et al*. 1996; Arana *et al*. 1999). Recently, ozone in different forms has also been used in a large number of medical indications (Finch *et al*. 1993; Alvarez *et al*. 1997; Morris and Menendez 1997; Falcon Lincheta *et al*. 1998; Komanapalli and Lau 1998). Ozone damages bacterial nucleic acids (Sawadaishi *et al*. 1986). Structural analysis of tRNA has shown that degradation occurs preferentially at guanine residues (Shinriki *et al*. 1981). Ozonolysis of supercoiled DNA has also been demonstrated (Sawadaishi *et al*. 1986), and both proteins and lipids are important targets in the reactions of ozone with bacterial membranes (Pryor and Uppu 1993). Ozone fractionates proteins at the tryptophan residues despite differences in amino acid and molecular weight (Pryor and Uppu 1993), whereas the reaction with lipids occurs at the carbon–carbon double bonds present in unsaturated fatty acid, producing different toxic products such as hydrogen peroxide, hydroxyhydroperoxides, aldehydes and Criegee ozonides (Pryor and Uppu 1993; Legnani *et al*. 1996).

The overuse of antibiotics in the treatment of infectious diseases, and the appearance of 'multi-drug resistant' bacterial strains (resistant to two or more antibiotics), has driven research towards the study of antimicrobial agents from essential oils (Hammer *et al*. 1999; Cox *et al*. 2000; Dorman and Deans 2000). Ozone does not contaminate the atmosphere and no bacterial resistance to this substance has been reported so far. Application of this system can be more extensive, ranging from the treatment of deep infections such as those caused by *Helicobacter pylori* and *Staphylococcus aureus* (Yamayoshi and Tatsumi 1993;

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Lezcano *et al.* 1998), to infection of the epidermis (Alvarez and O'Brien 1982). Different ozonized solutions have been used successfully against different infections such as otitis, intraocular infections and vaginitis (Finch *et al.* 1993; Gundarova *et al.* 1996; Morris and Menendez 1997).

Sunflower ozonized oil (Oleozon) has remarkable bactericidal properties as reported by Lezcano *et al.* (1998) in a preliminary study, and acts directly on the pathogenic micro-organism without damaging the human epithelium (Shinriki *et al.* 1981).

The Cuban 'Centro de estudio the medicamento CECMED' (Centro para el Control Estatal de la calidad de los Medicamentos, which has similar functions to the Food and Drug Administration of the USA) approved the Registration of Oleozon in 1999 (no. 1498) after the pre-clinical trial of Martínez Sanchez *et al.* (1997), which tested the dermal toxicity of Oleozon in rabbits and mice (Martínez Sanchez *et al.* 1997). In mice, they established that 2000 mg of Oleozon kg^{-1}, applied to the epithelium, did not produce toxic effects. All other parameters tested (weight, feeding, pineal reflex, motor ability etc.) were similar to the controls. Oleozon was seen to be slightly irritant, but all histological parameters (liver damage, kidney damage, biochemical parameters) were normal. The action of Oleozon on rabbits was comparable with that on mice.

The stability of Oleozon at different temperatures was also determined. Oleozon is stable for up to 1 year in the temperature range −10 to +8°C. Moreover, it is stable for up to 6 months at room temperature (27–30°C); after this period, the antimicrobial properties diminish. The pH is also stable for up to 1 year in the temperature range −10 to +8°C. At 30°C, the pH is stable for up to 6 months. The MIC values after this period increase from 2·37 to 19 mg ml^{-1}.

The purpose of this study was to investigate Oleozon activity against different bacteria, such as Mycobacteria and multi-drug-resistant Gram-positive and Gram-negative strains, isolated from different sites (skin, pus, eyes, stools). The safety of Oleozon at the sites from which the test strains were isolated was reported previously (Gundarova *et al.* 1996; Alvarez *et al.* 1997; Martínez Sanchez *et al.* 1997; Morris and Menendez 1997; Falcon Lincheta *et al.* 1998; Molerio *et al.* 1999).

MATERIALS AND METHODS

Sunflower oil ozonization

The sunflower oil was ozonized at the Centro de Investigaciones del Ozone (CENIC, Havana, Cuba) by an ozone generator Aqozo Industrial Ozonizer (Ozone Research Center, Cuba). Standardization of the preparation was carried out according to the following parameters:

- Peroxide Index (IP), which indicates the quantity of peroxide within the Oleozon. It is defined as the quantity of active oxygen per kilogram of Oleozon (mmol kg^{-1}) (Molerio *et al.* 1999). A range of IP between 500 and 800 (mmol kg^{-1}) was considered. The best antimicrobial activity was seen with an IP of 650 (mmol kg^{-1}).
- Acidity Index, which indicates the free fatty acid in the Oleozon. It is defined as the number of milligrams of potassium hydroxide that are necessary to neutralize the free fatty acid in 1 milligram of Oleozon (Panreac 1992). In sunflower oil, the value must range between 6 and 8 units (Vajdia and Saenz 1976) whereas in Oleozon, it is not above to 25 units (Molerio and Diaz 1999).
- Aldehyde concentration. The aldehyde concentration is measured by adding free hydroxylamine to the aldehyde carboxylic group. The results are expressed in mmol g^{-1} of Oleozon (Molerio and Diaz 1999); the interval must range between 0·4 and 0·9 mmol g^{-1}.
- Iodine Index, which is a measure of the unsaturation rate of sunflower oil expressed as the number of grams of iodine that react with 100 grams of sunflower oil. In sunflower oil, the rate varies between 125 and 135 units (Vajdia and Saenz 1976) whereas in Oleozon, the value is between 50 and 90 units (Molerio and Diaz 1999).
- Viscosity, which is a measure of the polymerization by condensation of the peroxides forming in the sunflower oil ozonization process. The values are expressed in mPa.s (centipoise or cP value; Institute of Standards and Technology). In order to obtain Oleozon with an IP between 500 and 800, the viscosity must be between 100 and 450 mPa.s (Molerio and Diaz 1999).

Bacterial strains

Different ATCC strains were tested: *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *E. coli* XL1, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus maleodoratus* ATCC 43197, *Ent. durans* ATCC 19432, *Ent. solitarius* ATCC 49428, *Ent. pseudoavium* ATCC 49372, *Ent. avium* ATCC 14025, *Ent. saccharolyticus* ATCC 43076, *Ent. hirae* ATCC 9790, *Ent. mundtii* ATCC 43186, *Ent. faecalis* ATCC 35038, Ent. gallinarum ATCC 49573, *Ent. casseliflavus* ATCC 25778, *Ent. faecium* ATCC 1974, *Mycobacterium tuberculosis* H37Rv and *M. smegmatis* mc^{155}.

Forty *Streptococcus pyogenes* strains were isolated from the skin of different patients attending the CENIC of Cuba; they were all tested for their susceptibility to Oleozon. Twenty-one *Ent. faecium* MDR strains, 27 *E. coli* MDR and other 13 *E. coli* strains, isolated at the 'Agostino Gemelli' Hospital in Rome (Sechi *et al.* 1998; Zanetti *et al.* 1998), and 50 strains of *Staph. aureus* isolated in Cuba, were also tested for their susceptibility to Oleozon. Nineteen *Staph. epidermidis* MDR strains were collected from ocular isolates of

patients attending the Institute of Ophthalmology of the University of Sassari (Sechi *et al*. 1999). Forty clinical *Ps. aeruginosa* strains isolated in Cuba were also evaluated. Three *M. tuberculosis* strains, two *M. avium* strains, two *M. chelonae* strains, two *M. fortuitum* strains and one *M. neoaurum* strain, isolated from different patients with different skin infections, were evaluated for their susceptibility to Oleozon.

MIC determinations

MICs were determined by the agar dilution method according to the NCCLS (1993) guidelines; the final inoculum was 10^4 cfu ml^{-1}. All susceptibility tests were repeated 10 times and were highly reproducible. For these experiments, Mueller Hinton Broth and Mueller Hinton Agar were obtained from Becton Dickinson. Doubling concentrations of Oleozon in the agar medium were used (19, 9·5, 4·75, 2·37 and 1·18 mg ml^{-1}). In order to mix the ozonized sunflower oil with the agar, 2 ml Tween-80 were added to 100 ml agar medium.

The MIC was defined as the lowest concentration of Oleozon inhibiting visible bacterial growth after incubation for 20 h at 37°C.

For Mycobacteria, the MIC of Oleozon was determined on solid medium by the microdrop agar proportion test. Middlebrook 7H9 broth and 7H10 were used as media (Bactec TB system, Becton Dickinson; Fadda and Roe 1984). A series of dilutions of the different strains of Mycobacteria was prepared in phosphate-buffered saline as a diluent. An aliquot (5 μl) of each dilution was spotted onto plates of 7H10 agar (Becton Dickinson), containing Tween-80 (to enhance oil solubility) and oleic acid albumin dextrose citric acid (OADC) as a supplement, and a series of dilutions from 19 to 1·18 mg ml^{-1} of Oleozon. The plates were incubated at 37°C (5 days for *M. smegmatis* and *M. neoarum*, 14 days for *M. avium*, 21 days for *M. tuberculosis*) and the number of bacterial colonies were counted. The MIC was defined as the lowest concentration resulting in a 99% reduction of the number of colonies on that plate compared with those on the plates used as controls (7H10 alone, and 7H10 plus Tween-80 and non-ozonized sunflower oil) for each dilution of the tested substance.

Quality control of potentially interfering substances

For each experiment, two bacterial spreads were performed as controls, one on the agar medium alone, and the second on the agar medium plus non-ozonized sunflower oil and Tween-80, for each concentration of ozonized oil. Bacterial growth was not inhibited by non-ozonized sunflower oil and Tween-80.

Quality control of agar plates

In order to verify the accuracy of the susceptibility test, a quality control programme was adopted as recommended by NCCLS (1993). Each batch of agar dilution plates was tested with the reference strain *E. coli* ATCC 25922 and Kanamycin as an antibiotic. All the MICs obtained were within the expected range (1–4 μg ml^{-1}).

RESULTS

Antimycobacterial activity

Under the test conditions, Oleozon showed an antimycobacterial activity against all strains evaluated (Table 1). The fast-growing strains *M. aurum* and *M. smegmatis* mc^{155} were both susceptible to Oleozon at a concentration of 0·95 mg ml^{-1}, as well as *M. tuberculosis* H37Rv. Oleozon was active against *M. abscessus* at a concentration of 2·37 mg ml^{-1}. *Mycobacterium fortuitum* strains showed an MIC ranging from 0·95 to 2·37 mg ml^{-1}, whereas Oleozon activity on both strains of *M. avium* analysed generated an MIC of 2·37 mg ml^{-1}. *Mycobacterium chelonae* strains were susceptible at 2·37 mg ml^{-1} Oleozon. *Mycobacterium tuberculosis* MIC ranged between 0·95 and 2·37 mg ml^{-1} (Table 1). In particular, the *M. tuberculosis* strain resistant to first line drugs (Rifampicin and Isoniazide) was susceptible to 2·37 mg ml^{-1} Oleozon.

Enterococci

Different *Enterococcus* ATCC strains were evaluated for their susceptibility to Oleozon (Table 2). Enterococci were susceptible from 2·37 to 9·5 mg ml^{-1}. *Enterococcus durans* ATCC 19432, *Ent. solitarius* 49428, *Ent. pseudoavium* ATCC 49372, *Ent. avium* ATCC 14025, *Ent. saccharolyticus* ATCC 43076, *Ent. hirae* ATCC 9790, *Ent. faecalis* ATCC 35038, *Ent. faecium* ATCC 19474 and *Ent. casseliflavus* ATCC 25778 were susceptible at a concentration of 9·5 mg ml^{-1}

Table 1 Susceptibility to Oleozon of different species of Mycobacteria

Species (no. of strains)	MIC (mg ml^{-1})
M. tuberculosis H37 Rv (1)	0·95
M. smegmatis mc155 (1)	0·95
M. abscessus (1)	2·37
M. aurum (1)	0·95
M. avium (2)	2·37
M. fortuitum (2)	0·95–2·37
M. chelonae (2)	2·37
M. tuberculosis (1)	0·95
M. tuberculosis MDR (2)	0·95–2·37

Table 2 Susceptibility to Oleozon of different enterococci, staphylococci, *Escherichia coli* and *Pseudomonas aeruginosa* ATCC strains

ATCC strains	MIC (mg ml^{-1})
Ent. hirae ATCC 9790	9·5
Ent. faecalis ATCC 35038	9·5
Ent. faecium ATCC 19474	9·5
Ent. gallinarum ATCC 49573	2·37
Ent. casseliflavus ATCC 25778	9·5
Ent. maleodoratus ATCC 43197	4·75
Ent. durans ATCC 19432	9·5
Ent. solitarius ATCC 49428	9·5
Ent. pseudoavium ATCC 49372	9·5
Ent. avium ATCC 14025	9·5
Ent. saccharolyticus ATCC 43076	9·5
Ent. mundtii ATCC 43186	2·37
Staph. aureus ATCC 29213	9·5
Staph. epidermidis ATCC 14990	2·37
E. coli ATCC 25922	4·75
E. coli XL1	1·18
Ps. aeruginosa ATCC 27853	4·75

Oleozon; *Ent. maleodoratus* ATCC 43197 generated an MIC of 4·75 mg ml^{-1} whereas *Ent. mundtii* ATCC 43186 and *Ent. gallinarum* ATCC 49573 generated an MIC of 2·37 mg ml^{-1}.

Oleozon showed good activity against 21 *Ent. faecium* clinical isolates (Table 3, MIC_{90}s ≤ 9·5 mg ml^{-1}). Three strains were susceptible at 9·5 mg ml^{-1}, and nine strains showed an MIC of 4·75 mg ml^{-1}; six strains generated an MIC of 2·37 mg ml^{-1} whereas three strains showed an MIC of 1·18 mg ml^{-1} Oleozon (Table 3). Ten clinical isolates of *Ent. faecalis* were also evaluated, and Oleozon was active at a concentration of 4·75–9·5 mg ml^{-1}.

Table 3 Susceptibility of different clinical isolates of *Enterococcus faecium*, *Ent. faecalis*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Staph. epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa*

	MIC (mg ml^{-1})		
Strains (no. of micro-organisms)	Range	50%	90%
Ent. faecium (21)	1·18–9.5	4·75	9·5
Ent. faecalis (10)	4·75–9·5	4·75	9·5
Strep. pyogenes (40)	2·37–9·5	4·75	9·5
Staph. aureus (50)	2·37–9·5	4·75	9·5
Staph. epidermidis (19)	2·37–9·5	4·75	9·5
E. coli (40)	1·18–9·5	4·75	9·5
Ps. aeruginosa (40)	4·75–9·5	4·75	4·75

Streptococci

Forty strains of *Strep. pyogenes*, isolated from the infected skin of Cuban patients, were evaluated for their susceptibility to Oleozon. The MIC range was from 2·37 to 9·5 mg ml^{-1} (Table 3).

Staphylococci

In Table 2, the activity of Oleozon against two *Staphylococcus* ATCC strains is shown: the MIC for *Staph. aureus* ATCC 25923 and *Staph. epidermidis* ATCC 14990 was 9·5 mg ml^{-1} Oleozon. Nineteen strains of *Staph. epidermidis* were also evaluated for their susceptibility to Oleozon (Table 3). MICs ranged from 2·37 to 9·5 mg ml^{-1}. Only four strains were susceptible at 2·37 mg ml^{-1} Oleozon. These strains were resistant to different drugs (strain 2 was resistant to penicillin, gentamicin and erythromycin; strain 7 was resistant to penicillin, tetracycline and erythromycin; strain 4 was resistant to penicillin and tetracycline; strains 6 and 18 were resistant to penicillin).

Escherichia coli

The activity of Oleozon against different *E. coli* strains is shown in Table 2. The MIC of *E. coli* XL1 was ≤ 1·18 mg ml^{-1}, whereas the MIC for *E. coli* ATCC 25922 was 4·75 mg ml^{-1}. The MIC for the clinical isolates was 4·75 mg ml^{-1}, except for three strains which showed an MIC of 2·37 mg ml^{-1}, and two strains with an MIC ≤ 1·18 mg ml^{-1}.

Pseudomonas aeruginosa

Pseudomonas aeruginosa ATCC 27853 was susceptible to 4·75 mg ml^{-1} (Table 2). Forty *Ps. aeruginosa* clinical isolates were also tested for their susceptibility to Oleozon (Table 3). All strains except one were susceptible at 4·75 mg ml^{-1}, one strain was susceptible at 9·5 mg ml^{-1}.

DISCUSSION

Pyogenic skin infections are produced in 90% of cases by *Staph. aureus* and *Strep. pyogenes*; *Ps. aeruginosa* and *E. coli* can participate as secondary agents (Alvarez *et al.* 1997; Falcon Lincheta *et al.* 1998; Neubert *et al.* 1999). Moreover, Gram-positive bacteria are rapidly becoming the most important pathogens in nosocomial infections. Recently, there has been great concern about multi-drug resistant *Staph. aureus*, *Staph. epidermidis*, *Ent. faecalis* and *Ent. faecium* strains (Yamayoshi and Tatsumi 1993; Esperson 1998). In this study, the action of Oleozon against different staphylococci, streptococci and enterococci is shown; some

of the strains were previously characterized by molecular methods (Sechi *et al*. 1998; Pinna *et al*. 1999).

Different forms of cutaneous tuberculosis are caused by various species of *Mycobacteria* (*M. tuberculosis*, *M. avium*, *M. fortuitum*, *M. chelonae*, *M. marinum* etc.) (Gulisano and Mariani 1998; Noguchi *et al*. 1998; Ena *et al*. 1999). In this study, *in vitro* activity of Oleozon against different bacteria (Mycobacteria, streptococci, enteroccoci, staphylococci, *E. coli* and *Ps. aeruginosa*) isolated from different sites (mostly skin infections) was evaluated. Oleozon showed a valuable antimicrobial activity against all micro-organisms tested. The activity of Oleozon (MIC range 1·18–9·5 mg ml^{-1}) against all tested bacteria, including β-lactam-, vancomicin- and gentamicin-resistant strains, is expressed in mg ml^{-1}. These concentrations may seem high if compared with the amount of antibiotics, expressed in μg ml^{-1}, necessary to inhibit bacterial growth. This is due to the dilution of the active compounds in the sunflower oil that has not been altered in the ozonization process. For Gram-negative bacteria, the activity of Oleozon was in the range 1·18–4·75 mg ml^{-1} (except for *Ps. aeruginosa* strains). Most of the *E. coli* strains tested showed an MIC of 4·75 mg ml^{-1}. Oleozon showed the same activity against *Ps. aeruginosa* ATCC strains. It was very effective against the slow-growing Mycobacteria tested in this study (*M. tuberculosis* and *M. avium*) and the fast-growing Mycobacteria (*M. aurum* and *M. smegmatis*). It was able to inhibit growth of these bacteria within a narrow range of concentration (0.95–2·37 mg ml^{-1}). Some *M. tuberculosis* multi-drug-resistant strains appeared less susceptible, with an MIC of 2·37 mg ml^{-1}, whereas other *M. tuberculosis* tested (H37Rv and a clinical isolate) generated an MIC of 0·95 mg ml^{-1}. The activity of Oleozon against the *M. avium*, *M. fortuitum* and *M. abscessus* strains tested was very efficient, with an MIC of 2·37 mg ml^{-1}. It seems from these preliminary results that Mycobacteria are even more susceptible to Oleozon than the other bacteria tested. This may be explained in part by the composition of their cell wall and the high lipid content, which may facilitate the passage of Oleozon-active compounds into the bacteria. It has been reported that Oleozon is effective in the treatment of different types of skin diseases caused by herpes, Mycobacteria, staphylococci, streptococci etc. (Ena *et al*. 1999; Gulisano and Mariani 1998; Neubert *et al*. 1999). For instance, Morris and Menendez (1997) successfully treated 180 patients affected by *Herpes simplex* infection in the lips with a twice-daily application of Oleozon.

Oleozon has also been applied for the cure of fungal infections; 213 patients with a mean age of 28 years were followed up. *Trichophytom rubrum*, *Candida albicans*, *Microsporum canis* and *Trichophytom mentagrophytes* were the isolates. Seventy-five percent of the patients were cured with topical applications; treatment with the antimycotic compound Nizoral on the same type of infections was effective for 81% of the patients (Falcon Lincheta *et al*. 1998).

Alvarez *et al*. (1997) reported a successful treatment of a patient with pyoderma by daily topical applications of Oleozon with an I.P. of 650.

The results obtained in this study should lead to the setting up of some clinical trials in order to compare the efficacy of Oleozon with other antimicrobial agents. The wide availability of sunflower oil makes Oleozon a competitive antimicrobial agent. The fact that most MICs were clustered around 2·37 mg ml^{-1}, and no strains were found with an MIC higher than 9·5 mg ml^{-1}, may suggest that the activity is due to toxicity rather than to metabolic interruption, as is the case for traditional antimicrobial agents. The fact that Oleozon showed no toxicity to rabbits and mice (Molerio and Diaz 1999), however, is an indication that it has multiple targets (i.e. membrane proteins) but shows no generalized toxicity to the cells.

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